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ASSAYS FOR NON-INSULIN DEPENDENT DIABETES

FIELD OF THE INVENTION

5

The present invention relates to screening methods using insulin receptor substrate molecules.

BACKGROUND OF THE INVENTION

10 In mammals, insulin is the principal hormone controlling blood glucose levels and acts by stimulating glucose intake and metabolism in muscle and adipocytes. Insulin action is mediated through the insulin receptor, a transmembrane glycoprotein with protein tyrosine kinase activity. Insulin binds to the α subunit of the insulin receptor, which activates the tyrosine kinase in the β subunit. The kinase activity appears to mediate the insulin response

15 through phosphorylation of the receptor itself and substrates like insulin receptor substrate-1 (IRS-1). In contrast to other protein tyrosine kinase receptors, which autophosphorylate and then bind to Src homology 2 (SH2) domain-containing proteins, the insulin receptor does not appear to bind directly to SH2 proteins. Rather, the insulin receptor phosphorylates IRS-1, which in turn recognize and bind to the SH2 domains of various signal transduction proteins,

20 including Syp, Nck, Grb2, Pyn, and the SH2 domain of the α subunit of the receptor itself. (See Myers, M. G., Jr., et al., *Proc. Natl. Acad. Sci. USA* 89:350-354 (1992), Myers, M. G., Jr., et al., *Mol. Cell. Biol.* 14:3577-3587 (1994), Noguchi, T., et al., *Mol. Cell. Biol.* 14:6674-6682 (1994), Kuhne, M. R., et al., *J. Biol. Chem.* 268:11479-11481 (1993), and White et al., *J. Biol. Chem.* 269:1-4 (1994), for a general discussion, and references cited therein).

IRS-1 deficient mice display impaired insulin-stimulated glucose disposal in vivo as well as glucose transport in vitro (Tamemoto, H., et al., *Nature* 372:182-186 (1994), Araki, E., et al., *Nature* 372:186-190 (1994)) but their survival and residual insulin sensitivity is dependent on the presence of IRS-2 (Sun, X-J., et al., *Nature* 377:173-177 (1995), Patti, M. E., et al., *J. Biol. Chem.* 270:24670-24673 (1995)).

5 Non-insulin dependent diabetes mellitus (NIDDM) is due in large part to insulin resistance, which occurs when the target cells no longer respond to ordinary levels of circulating insulin. However, little is known about the roles of regulation of IRS proteins normal physiology or 10 in insulin-resistant states in human subjects. Thus, it would be highly desirable to discover a correlation between NIDDM and a biochemical mechanism.

SUMMARY OF THE INVENTION

15 The present invention provides methods for screening for a bioactive agent capable of binding to an insulin receptor substrate (IRS) molecule. The method comprises adding a candidate bioactive agent to a sample of the IRS molecule. The presence or absence of binding of the candidate agent to the IRS molecule is then determined.

20 In an additional aspect, the present invention provides methods for screening for a bioactive agent capable of modulating the activity of an IRS molecule. The method comprises adding a candidate bioactive agent to a cell population. The activity of the IRS molecules in the cell population is then measured.

25 The invention also provides bioactive agents identified using the methods of the invention.

30 In a further aspect, the present invention provides methods of diagnosing individuals at risk for type II diabetes mellitus. The method comprises measuring the amount of IRS-1 in adipocytes in a first individual, and comparing the amount to an amount of IRS-1 in adipocytes from a second unaffected individual. When the activity of IRS-1 from the first

individual is less than the activity of IRS-1 in the second individual, the first individual is at risk for type II diabetes mellitus.

DETAILED DESCRIPTION OF THE INVENTION

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The present invention is based on the discovery that IRS-1 levels in non-insulin dependent diabetes mellitus (NIDDM) individuals are significantly reduced (50-90%) as compared to the levels in comparable healthy individuals. This provides for the first time both a potential diagnostic tool for NIDDM as well as a target for candidate drug screening to elevate the 10 levels of either IRS-1 or IRS-2. Prior to this discovery, no correlation between NIDDM and a biochemical characteristic such as protein expression was known. Thus, the present invention allows diagnostic assays for NIDDM, and, importantly, target molecules for use in screening drug candidates.

15 Accordingly, the present invention provides methods for screening candidate bioactive agents which are capable of modulating the activity of IRS-1 and IRS-2 (collectively, IRS molecules). That is, since the present discovery that individuals with NIDDM have decreased levels of IRS-1 expression, bioactive agents that increase the levels or activity of IRS-1 are desirable. Similarly, since previous work has shown that IRS-2 can, at least in part, 20 compensate for low levels of IRS-1, bioactive agents that increase the levels or activity of IRS-2 may also be desirable.

Thus, the present invention is directed to methods of screening candidate bioactive agents. By "candidate bioactive agents" or "candidate drugs" or grammatical equivalents herein is 25 meant any molecule, e.g. polypeptides (including proteins and peptides), small organic or inorganic molecules, polysaccharides, polynucleotides, etc. which may be tested for the ability to directly or indirectly bind to either or both IRS-1 or IRS-2, and/or preferably modulate their activity, as defined below. Candidate agents encompass numerous chemical classes. For example, since increasing transcription of IRS-1 or IRS-2 may increase the total 30 activity present in a cell, preferred candidate agents include known transcription factors or

transcription factor candidates, including both proteinaceous and nucleic acid (usually RNA) transcription factors. In another embodiment, the candidate agents are organic molecules, particularly small organic molecules, comprising functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an
5 amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more chemical functional groups.

10 Candidate agents are obtained from a wide variety of sources, as will be appreciated by those in the art, including libraries of synthetic or natural compounds. Any number of techniques are available for the random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are
15 available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications to produce structural analogs.

20 Candidate bioactive agents may be assayed or screened in a number of ways. In one embodiment, candidate bioactive agents may be preliminarily screened for their ability to directly bind to IRS-1 or IRS-2, using techniques well known in the art. These techniques may include, but are not limited to, labeled *in vitro* protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. By
25 "labeled" herein is meant a compound that has at least one element, isotope or chemical compound attached to enable the detection of the compound. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) immune labels, which may be antibodies or antigens; and c) colored or fluorescent dyes. The labels may be incorporated into the compound at any position.

The binding assays utilize IRS molecules. Preferably, the IRS molecule is from human, although other IRS proteins may be used. For example, it is known that the IRS-1 molecules is highly conserved between species. Thus, for example, IRS molecules (both IRS-1 and IRS-2) from other species may be used, preferably other mammalian species such as rat and

5 mouse.

The IRS proteins are made using techniques well known in the art. For example, recombinant IRS nucleic acids are utilized to make recombinant IRS proteins. The sequence of both IRS-1 and IRS-2 are known (see for example Sun et al., Nature 377:173 (1995), and references cited therein, all of which are expressly incorporated by reference herein). A "recombinant protein" is a protein made using recombinant techniques, i.e. through the expression of a recombinant nucleic acid. A recombinant protein is distinguished from naturally occurring protein by at least one or more characteristics. For example, the protein may be isolated or purified away from some or all of the proteins and compounds with which it is normally associated in its wild type host, and thus may be substantially pure. For example, an isolated protein is unaccompanied by at least some of the material with which it is normally associated in its natural state, preferably constituting at least about 0.5%, more preferably at least about 5% by weight of the total protein in a given sample. A substantially pure protein comprises at least about 75% by weight of the total protein, with at least about 15 80% being preferred, and at least about 90% being particularly preferred. The definition includes the production of a IRS protein from one organism in a different organism or host cell. Alternatively, the protein may be made at a significantly higher concentration than is normally seen, through the use of a inducible promoter or high expression promoter, such that the protein is made at increased concentration levels. Alternatively, the protein may be in a 20 form not normally found in nature, as in the addition of an epitope tag, a purification signal such as His₆, or amino acid substitutions, insertions and deletions, as discussed below.

25 Using IRS nucleic acids which encode IRS proteins, a variety of expression vectors are made. The expression vectors may be either self-replicating extrachromosomal vectors or vectors which integrate into a host genome. Generally, these expression vectors include

transcriptional and translational regulatory nucleic acid operably linked to the nucleic acid encoding the IRS protein. "Operably linked" in this context means that the transcriptional and translational regulatory DNA is positioned relative to the coding sequence of the IRS protein in such a manner that transcription is initiated. Generally, this will mean that the 5 promoter and transcriptional initiation or start sequences are positioned 5' to the IRS protein coding region. The transcriptional and translational regulatory nucleic acid will generally be appropriate to the host cell used to express the IRS protein; for example, transcriptional and translational regulatory nucleic acid sequences from *Bacillus* are preferably used to express the IRS protein in *Bacillus*. Numerous types of appropriate expression vectors, and suitable 10 regulatory sequences are known in the art for a variety of host cells.

In general, the transcriptional and translational regulatory sequences may include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. In a 15 preferred embodiment, the regulatory sequences include a promoter and transcriptional start and stop sequences. In addition, the expression vector may comprise additional elements, including, for example, two replication systems, thus allowing it to be maintained in two organisms; at least one sequence homologous to the host cell genome to allow recombination, for integrating vectors; and/or selectable marker genes to allow the selection of transformed 20 host cells.

The IRS proteins of the present invention are produced by culturing a host cell transformed with an expression vector containing nucleic acid encoding a IRS protein, under the appropriate conditions to induce or cause expression of the IRS protein. The conditions 25 appropriate for IRS protein expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation.

The IRS proteins may be produced in a number of cell types, including, but not limited to, 30 yeast, bacteria, archebacteria, fungi, and insect and animal cells, including mammalian cells.

Of particular interest are *Drosophila melanogaster* cells, *Saccharomyces cerevisiae* and other yeasts, *E. coli*, *Bacillus subtilis*, SF9 cells, C129 cells, 293 cells, Neurospora, BHK, CHO, COS, HeLa cells, and adipocytes.

5 The IRS protein may also be made as a fusion protein, using techniques well known in the art. Thus, for example, the IRS protein may be made as a fusion protein to increase expression, for ease in purification, or for other reasons. Also included within the definition of IRS proteins of the present invention are amino acid sequence variants. These variants fall into one or more of three classes: substitutional, insertional or deletional variants. These

10 variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the IRS protein, using cassette or PCR mutagenesis or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture as outlined above. However, variant IRS protein fragments having up to about 100-150 residues may be prepared by *in vitro* synthesis using established

15 techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the IRS protein amino acid sequence. The variants typically exhibit the same qualitative biological activity as the naturally occurring analogue, although variants can also be selected which have modified characteristics as will be appreciated by those in the art.

20 In a preferred embodiment, the IRS protein is purified or isolated after expression for use in the binding assays of the invention. IRS proteins may be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample. Standard purification methods include electrophoretic, molecular, immunological

25 and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, and chromatofocusing. For example, the IRS protein may be purified using a standard anti-IRS antibody column. If purification sequences are included, such as the His₆ tag, suitable methods are used, such as a metal-containing column. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also

useful. For general guidance in suitable purification techniques, see Scopes, R., *Protein Purification*, Springer-Verlag, NY (1982).

Once expressed and purified if necessary, the IRS proteins are used in screening assays. In 5 this embodiment, candidate bioactive agents are added to a sample of IRS-1 or IRS-2, made as described above, and then the mixture is evaluated to determine whether the candidate agent binds to the IRS molecules to form a complex. This is generally done by using either labeled candidate agent or labeled IRS molecules, with the unbound species being separated or purified away, to allow detection of the complex. Thus, for example, either the IRS 10 molecules or the candidate agents may be bound to an insoluble support having isolated sample receiving areas (e.g. a microtiter plate). That is, in one embodiment, the IRS molecules are attached to the insoluble support, and labeled candidate agents are added, the excess unbound material is washed away, and detection of agent binding to the IRS molecule is done via detection of the label. Alternatively, the candidate agents may be bound to the 15 support (they may even be synthesized on the support) and then labeled IRS molecules are added and assayed as above.

The insoluble supports may be made of any composition to which the agents and/or IRS molecules may be bound, are readily separated from soluble material, and are otherwise 20 compatible with the overall method of screening. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports include surface arrays such as are known in the art, microtiter plates, membranes and beads. These are typically made of glass, plastic (e.g. polystyrene), polysaccharides, nylon or nitrocellulose, which have some sort of suitable binding functionalities on the surface. 25 Microtiter plates and surface arrays are especially convenient because a large number of assays may be run simultaneously, using small amounts of reagents and samples. The particular manner of binding of the agent and/or IRS molecules is not critical as long as it is compatible with the reagents and other components of the system, maintains the activity of the agent or IRS molecule and is nondiffusible. Preferably, the assays are conducted under 30 approximately physiological conditions. Following binding of the agent and/or IRS

5 molecules, excess unbound material is removed by washing. Any unreacted binding functionalities may be blocked, if necessary, as is known in the art, for example through the addition of neutral or carrier proteins (e.g. bovine serum albumin) so as to prevent direct binding of the other component of the complex to the insoluble support rather than forming a complex.

10 Once the candidate agents or IRS molecules are bound to the insoluble support, the other component is added, preferably in a labeled form (i.e. if candidate agents are bound to the support, the labeled IRS molecules are added; if IRS molecules are bound to the support, labeled candidate agents are added). Alternatively, it is also possible to add the label afterwards; for example, after the addition of IRS molecules to bound candidate agents, it is possible to add labeled antibodies or other binding moieties, to the IRS molecules. Thus, for example, other proteins known to bind to the IRS molecules may be labeled and used to detect the IRS molecules. Incubation of the samples is for a time sufficient to allow binding 15 to occur. Following incubation, all samples are washed free of non-specifically bound material and the amount of bound, labeled material determined. Suitable positive and negative controls are also preferably run.

20 The present invention is directed also to drug screening assays. Drug screening assays utilize the discovery that NIDDM patients have decreased amounts of IRS-1 proteins to identify agents that modulate the activity or amount of IRS-1 or IRS-2. The method comprises the steps of adding or contacting a candidate bioactive agent to or with a cell, and measuring the activity of IRS-1 in the cell.

25 In a preferred embodiment, the present invention is directed to methods of screening bioactive agents capable of modulating the activity of IRS molecules, i.e. IRS-1 and IRS-2. By "modulating the activity" herein is meant that the biological activity of the protein is altered, i.e. either increased or decreased. In a preferred embodiment, the biological activity is increased. This may be done in several ways, for example, the total amount of the protein 30 may be increased, i.e. the expression of the protein is increased, and thus the measured

biological activity increases due to more protein being present. This may be done either by increasing the level or rate of transcription and/or translation of the protein in the cell. Alternatively, the specific activity of the protein may be increased; that is, the protein is made more active by the presence of the bioactive agent, and thus an increase in the total biological 5 activity is seen.

In an alternative embodiment, the activity of the protein is decreased. This may be important to help elucidate the mechanism of action, for example, or to provide structural information about the sites of protein interaction of the molecule. Similarly, agents which decrease the 10 level of activity of the protein may be useful to define further candidate bioactive agents for testing.

The candidate agents are added or contacted with the cells. Suitable cells for use in the invention include, but are not limited to, adipocytes. Preferred cells include adipocytes from 15 either healthy or NIDDM individuals, with cells from NIDDM individuals being preferred. In a preferred embodiment, cells from NIDDM individuals that show a large decrease in the amount of IRS-1 protein are used (e.g., 90%); in other embodiments, testing may be done with cells from several different individuals.

20 Tissue is surgically harvested from patients using well known techniques, and the adipocytes isolated as is known in the art (see Smith et al., J. Lipid Res. 13:822-827 (1972), hereby expressly incorporated by reference). This is generally done using collagenase to separate the cells. The cells may then be used in the assays.

25 In one embodiment, the cells are immortalized, i.e. transformed to form cell lines. Methods suitable to make cell lines are well known in the art.

A variety of additional reagents, in addition to the cells and candidate agents, may be 30 included in the screening assay. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc., which may be used to facilitate optimal protein binding and/or

reduce non-specific or background interactions. Also reagents that otherwise improve the efficacy of the assay, such as inhibitors (protease, nuclease, etc.), anti-microbial agents, etc. may be used.

5 The mixture of components may be added in any order that provides for the interaction of the candidate agent and the cell. In some embodiments, the candidate agents may be accompanied by or fused to targeting or signaling molecules that facilitate the uptake or targeting of the agent to different cellular localizations. For example, endocytosis signals, nuclear localization signals, or other molecules for localization in the Golgi, endoplasmic

10 reticulum, nucleoli, nuclear or cellular membrane, mitochondria, secretory vesicles, lysosome, etc. Thus, for example, when the candidate agents are candidate nucleic acid transcription factors or analogs, nuclear localization signals are preferably used. The components are allowed to incubate with the cells under suitable thermal conditions, generally between 4°C and 40°C, with physiological temperatures (about 37°C) being

15 preferred, for a suitable amount of time. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening.

Once the candidate agent is added or contacted with a cell, the activity of the IRS molecule is measured. The modulation of the activity of the protein is measured in any number of ways, as will be appreciated by those in the art. For example, the activity is measured by measuring the glucose-transport activity in response to different concentrations of insulin, as is outlined in the Examples and described in Rondinone et al., *J. Biol. Chem.* 271:18148-18153 (1996), hereby expressly incorporated by reference. Briefly, the cells are incubated with different doses of insulin in the presence of radiolabelled glucose for a period of time, generally from about 15 minutes to 2 hours, with about 1 hour being preferred. The excess glucose is removed, generally via centrifugation through silicone oil, and the radioactivity is measured by scintillation counting. As is shown in the Examples, individuals with NIDDM exhibit a decrease in insulin response, relative to non-NIDDM individuals. Thus, for example, normal individuals generally exhibit about a 300% increase over basal levels of glucose transport in the presence of maximal concentrations of insulin. NIDDM individuals, in contrast,

generally exhibit about a 50% increase over basal levels. Thus, when NIDDM cells are used in the assay, candidate agents which increase the insulin effect by greater than about 50% over basal transport are desired.

5 Alternatively, activity is measured as a function of amount of protein; that is, the activity is measured by measuring the amount of IRS-1 or IRS-2 protein, using techniques well known in the art. In one embodiment, the methods utilize immunoprecipitation techniques, such as are outlined in the examples, to determine the amount of protein. In a preferred embodiment, the methods utilize enzyme-linked immunosorbent assays (ELISA) techniques for the
10 detection and quantification of the proteins. ELISA techniques are well known in the art; see Ausubel et al., Short Protocols in Molecular Biology, 3rd Edition, John Wiley & Sons, Inc., 1995. Assays to determine affinity and specificity of binding are known in the art, including competitive and non-competitive assays. Assays of interest include ELISA, RIA, flow cytometry, etc.

15 In a preferred embodiment, the activity of the IRS molecule may be measured prior to the addition of the candidate agent. Generally, this is done periodically on the cells or cell line being used, and thus may not need to be done prior to every assay. That is, generally the starting range of IRS activity is initially measured and then known, with the appropriate
20 controls being run as necessary. Alternatively, negative controls may be run with every batch of candidate agents. Alternatively, the activity of the IRS molecule is determined prior to the addition of the candidate agents.

25 The level of IRS activity after the addition of the candidate agent is then compared to the level of activity prior to the addition. If the post-addition activity is different than the initial activity, the candidate agent is capable of modulating the activity of the IRS molecule. As outlined above, this difference may be either an increase or a decrease, and generally will be at least about 5% different, with at least about 10% different being preferred, at least about 20% being particularly preferred and at least about 40-50% being especially preferred.

In a preferred embodiment, the present invention provides methods for diagnosing individuals at risk for type II diabetes mellitus, i.e. NIDDM, or for confirming a diagnosis of NIDDM.

The fact that levels of IRS-1 are decreased in NIDDM individuals, as compared to non-NIDDM individuals, allows for the screening of individuals for NIDDM. This may confirm a 5 diagnosis of NIDDM in an individual affected, i.e. showing the symptoms of NIDDM.

Alternatively, the screen may serve to identify individuals who may not have the full-blown symptoms of NIDDM but may be at risk to develop such symptoms in the future, as outlined in the examples:

10 Thus, in a preferred embodiment, adipocytes are obtained from a patient, as is known in the art, for example using needle biopsies, and purified if necessary. The amount of IRS protein is then determined, using techniques known in the art and outlined herein, and compared to IRS levels in a matched, healthy individual. Decreases in IRS protein, preferably IRS-1 protein, of about 50%-90% have been shown to correlate with NIDDM or prediabetic 15 conditions as outlined in the Examples. Thus, in a preferred embodiment, a reduction in IRS levels of at least about 40%-50%, more preferably about 60%-70%, and more preferably about 80%-90%, as compared to healthy matched individuals, is indicative of patients either at risk for type II diabetes or with the disease. Generally, a decrease of at least about 50% in IRS-1 levels is indicative of these conditions.

20 In a preferred embodiment, the methods of the invention allow the diagnostic differentiation between insulin dependent diabetes mellitus (IDDM) and NIDDM. Thus, patients which exhibit diabetic symptoms can be characterized as either IDDM or NIDDM based on their 25 levels of IRS proteins. Similarly, as shown in the examples, these tests allow the correct identification of the diabetic type, to verify or correct initial diagnosis.

Accordingly, the method comprises measuring the activity of IRS-1 in adipocytes from an individual being tested, and comparing this result to the activity of IRS-1 in adipocytes from a second, unaffected individual, preferably matched as a control individual. In a preferred 30 embodiment, the amount of IRS-1 is measured, using the techniques outlined above or others

known in the art. When the activity from the test subject is less than the activity from the unaffected subject, the test subject is at risk for NIDDM, and in fact may already have it. As outlined above, a 50% or greater reduction in IRS levels is the indicator of status.

5 The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference.

10

EXAMPLES

15 **Glucose transport in human adipocytes.** Specimens of human subcutaneous adipose tissue were obtained from the abdominal region of non-diabetic subjects (n=14), NIDDM (n=12), or IDDM (n=8) subjects. The healthy and NIDDM subjects were of similar age and degree of obesity (BMI). The biopsies were placed in Medium 199 at 37°C containing 25 mM Hepes, 4% BSA with 5.5 mM glucose. The study was approved by the Ethical Committee of the Gotsborg University. Adipose cells were prepared according to methods previously described (Smith, U., et al., *J. Lipid Res.* 13:822-827 (1972)). Adipocytes were isolated by 20 digesting about 0.6 g of tissue for 50 min at 37°C in Medium 199 containing 25 mM Hepes, 4% BSA, 5.5 mM glucose and 0.8 mg/ml collagenase (Sigma) in a shaking water bath. Cells were incubated for 15 min in the absence or presence of the indicated concentrations of 25 human insulin (Novo Nordisk) in the presence of 0.1 μM N6(2-Phenylisopropyl)-adenosine (PIA) and 1 U/ml adenosine deaminase. Glucose transport activity was assayed for 1 h using 0.86 μM ¹⁴C-U-glucose (Amersham) as described (Rondinone, C. M., & Smith, U., *supra*). The cells were separated from the incubation medium by centrifugation through silicone oil and the radioactivity associated with the cells was measured by scintillation counting.

30 The results were as follows. The half-maximal response in healthy subjects was seen at 0.23 nM and the maximal (~300% increase over basal) at 0.35 nM insulin. In contrast, in cells

from NIDDM patients, both the concentration required to significantly increase glucose transport and the maximal response were significantly impaired with only 50% increase with respect to basal transport at maximal concentrations of insulin.

5 **Immunoprecipitations and Immunoblotting.** To determine whether the impairment in insulin action on NIDDM adipocytes was associated with an abnormality in proteins involved in insulin signalling, immunoblotting was performed with antibodies against several proteins. Isolated human adipocytes were distributed into plastic vials (12-15% cell suspension) in a final incubation volume of 400 μ l. Cells were preincubated with or without 6.9 nM insulin
10 for 10 min, immediately separated by centrifugation through silicone oil and lysed in 0.4 ml lysis buffer containing 25 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 25 mM NaCl, 1% Nonidet P-40, 1 mM Na₃VO₄, 10 mM NaF, 0.2 mM leupeptin, 1 mM benzamidine and 0.5 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochlorine (AEBSF) and rocked for 40 min at 4°C. Detergent-insoluble material as sedimented by centrifugation at 12,000xg for 10 min at
15 4°C. Cell lysate (50ug protein) was run on the gel or 100 ug protein were immunoprecipitated for 2 h with the anti-IRS-1 C-terminal (4 μ g/ml (UBI) or anti-IRS-2 (3 μ l/ml) antibodies. Antibodies against IRS-2 were prepared in rabbits using an IRS-2 -specific peptide comprised of amino acids 1310-1322 (LSHHLKEATVVKE). Immune complexes
20 were collected with Protein A-sepharose, washed, solubilized in Laemmli sample buffer and separated using 7.5% SDS-PAGE. Proteins were transferred from the gel to nitrocellulose sheets and blocked in 5% milk. The blots were probed with the different primary antibodies: anti-IRS-1 C-terminal, anti-p85 (whole antiserum), anti-SHPTP2/syp, anti-Grb2 and 4G10 anti-PY antibodies (UBI); anti-IR (Transduction Laboratories), anti-IRS-1 (NH-2 terminal), anti p110 (Santa Cruz Biotechnology) according to the recommendations of the manufacturer
25 or anti-IRS-2 (1:500 v/v) and the proteins were detected by enhanced chemiluminescence using horseradish peroxidase-labeled second antibodies (Amersham). The intensity of the bands was quantitated with a laser densitometer (Molecular Dynamics). Quantification was also verified using ¹²⁵I-Protein A. For PI 3-kinase assays immunoprecipitates were washed extensively and the PI 3-kinase reaction was performed as described (Rondinone, C. M., &
30 Smith, U., *supra*).

PI 3-Kinase Activity: PI 3-kinase assay was performed directly on the immunoprecipitates as described (Rondinone et al., *J. Biol. Chem.*, 271:18148 (1996); Auger et al., *Cell* 57:167 (1989), both of which are expressly incorporated by reference). Briefly, 6 μ l of a mixture of PI (10 μ g/sample) and phosphatidylserine (2.5 μ g/sample) were added to the beads and the 5 reaction was started by the addition of 30 μ l of a reaction mixture consisting of 40 mM Hepes (pH 7.5), 20 mM MgCl₂, and 50 μ M [γ ³²P]ATP (0.2 μ Ci/ μ l; 1 Ci = 37 GBq). After 15 min at 30°C, the reaction was stopped by the addition of 40 μ l of 4 M HCl and 160 μ l of 10 CHCl₃/CH₃OH (1:1). The organic phase was extracted and applied to a silica gel thin layer chromatography plate precoated with 1% potassium oxalate (Analtech). The chromatography plates were developed in CHCl₃/CH₃OH/H₂O/NH₄OH (60:47:11.3:2), dried, and visualized by autoradiography. The radioactivity was quantitated with a PhosphorImager (Molecular Dynamics).

The results showed that IRS-1 was reduced by 50 to 90% (average, 70 \pm 6%) in adipocytes 15 from NIDDM patients compared to those from non-diabetic subjects. This was confirmed by using two different NG2- and C-terminal antibodies. However, the insulin receptor, the p85 subunit of PI-3 kinase and the phosphotyrosine phosphatase SHPTP2/Syp were unchanged. Similarly, the IRS-1 protein content was not reduced in adipocytes from IDDM subjects. As 20 expected, in IRS-1 or PI-3 kinase (p85 and p110 subunits) in response to insulin could hardly be detected. Despite the big reduction of IRS-1, immunoblots using antiphosphotyrosine antibodies in whole cell lysates from basal or insulin-stimulated NIDDM adipocytes showed a tyrosine phosphorylated protein at MW-185 kDa suggesting the presence of another protein at that MW. The likely candidate was the recently identified docking protein IRS-2 (Sun, X-J., et al., *Nature* 377:173-177 (1995)). In immunoprecipitates using polyclonal IRS-2 25 antibodies, IRS-2 was present in a similar extent in adipocytes from both NIDDM and healthy subjects; IRS-2 was also tyrosine phosphorylated in an insulin-dependent fashion. It was clear that both PI-3 kinase and Grb2 became associate with IRS-2 in response to insulin as also previously found (Patti, M. E., et al., *supra*, Tobe, K., et al., *J. Biol. Chem.* 270:5698-5701 (1995)). Supernatants from anti-IRS-2 immunoprecipitations analyzed by 30 immunoblotting, again showed that IRS-1 was barely detectable in NIDDM cells.

The PI 3-kinase activity recovered in IRS-2 and IRS-1 immunoprecipitates was markedly stimulated in normal adipocytes by insulin. Again, little or no PI 3-kinase activity was recovered in IRS-1 immunoprecipitates from NIDDM adipocytes. In these cells, PI 3-kinase activity was virtually exclusively recovered in the anti-IRS-2 immunoprecipitates.

5 The concentration-dependent ability of insulin to increase the tyrosine phosphorylation of IRS-1 and IRS-2 and the subsequent binding of PI 3-kinase in adipocytes from healthy subjects was also examined. Both IRS-1 and IRS-2 became tyrosine phosphorylated by insulin but quantification of the immunoblots showed that the effect of a maximal insulin
10 concentration was 50% greater for IRS-1 than for IRS-2. Therefore, immunoprecipitates with anti-IRS-1 or anti-IRS-2 antibodies showed that at all insulin concentrations IRS-1 was the main docking protein for PI 3-kinase. However, IRS-2 could also bind PI 3-kinase but this was, for a given insulin concentration, less (~40%) than that bound to IRS-1. This provides evidence for differences between IRS-1 and IRS-2 that may be related to a different
15 interaction between IRS-1 and IRS-2 and the insulin receptor (He, W., et al., *J. Biol. Chem.* 271:11641-11645 (1996), Sawka-Verhelle, D. J., et al., *J. Biol. Chem.* 271:5980-5983 (1996)). This was further verified when the PI 3-kinase lipid kinase activity was measured. At all insulin concentrations, IRS-1 accounted for most (~70%) of the IRS-associated PI 3-kinase activity in response to insulin in adipocytes from normal subjects. In contrast, IRS-2
20 was the main source of PI 3-kinase activity in adipocytes from NIDDM subjects.

Thus, this study demonstrated that IRS-1 protein expression is markedly reduced in adipocytes from NIDDM subjects in comparison with both healthy subjects and individuals with IDDM. The 50% or greater reduction in IRS-1 content was consistently seen in the 12
25 NIDDM subjects studied. So far, more than 20 NIDDM patients have been examined and only a smaller reduction (~30%) was seen in two subjects. This reduction can not be accounted for by obesity since the control and NIDDM subjects had a similar BMI or by hyperinsulinemia/hyperglycemia since the IDDM were unaffected. In contrast, IRS-2 levels were unchanged in NIDDM cells. Skeletal muscle from morbidly obese individuals has also
30 been shown to have a moderate reduction in IRS-1 protein content (Goodyear, L., et al., *J.*

Clin. Inves. pp 2195-2204 (1995) but it is not clear whether this reduction was confined to subjects with an impaired glucose tolerance.

IRS-1 seems to be the main docking protein for PI 3-kinase and the associated increase in 5 glucose uptake in normal human adipocytes as also demonstrated in other cells (Quon, M. J., et al., *Biol. Chem.* 269:27920-27924 (1994), Har, K., et al., *Proc. Natl. Acad. Sci. USA* 91:74155-7419 (1994), Quon, M. J., et al., *Mol. Cell. Biol.* 15:5403-5411 (1995)). However, similar to the IRS-1 deficient mice (Tamemoto, H., et al., *supra*., Araki, E., et al., *Nature* 372:186-190 (1994), Patti, M. E., et al., *supra*.), IRS-2 seems to be able to replace IRS-1 as 10 the main docking protein for binding and activation of PI 3-kinase. However, in human adipocytes, IRS-2 requires a higher insulin concentration than IRS-1 for similar binding and activation of PI 3-kinase. This is in parallel to the ability of insulin to increase total tyrosine phosphorylation which also was reduced in IRS-2. These findings may provide important 15 reasons for the insulin resistance in NIDDM.

15 The basic mechanism(s) for the reduction in IRS-1 expression in NIDDM is currently unclear. However, preliminary evidence indicates that IRS-1 mRNA levels are reduced in adipocytes from NIDDM subjects. Whether this is sufficient to account for the low IRS-1 content or whether there is also an increased protein degradation is unclear.

20

Example 2 Further Studies

25 64 additional patients were evaluated as follows. 20 obese and healthy individuals, 22 healthy young individuals with massive diabetes heredity factor (at least 2 first degree relatives with NIDDM), and 22 matched and healthy individuals without known diabetes heredity were tested for IRS-1 levels, using needle biopsies to obtain adipocytes. Of these, 6 of the 20 obese individuals showed IRS-1 levels that were decreased by at least 50%, with IRS-2 levels that remained constant. Of these 6, two were found to have previously unknown 30 NIDDM, 2 more had reduced glucose tolerance, and 1 patient had a massive insulin

resistance, which is the precursor to NIDDM. Furthermore, the lowered IRS-1 levels were six times more commonly seen among the healthy group with known diabetic heredity than in the control group. In addition, individuals that exhibit a lowered IRS-1 level are more insulin resistant than those without. Finally, a simultaneous lowered level of IRS-1 and lowered 5 levels of GLUT 4 transporter protein appears to be a particularly strong indicator to define the prediabetic group.

CLAIMS

We claim:

1. A method for screening for a bioactive agent capable of binding to an insulin receptor substrate (IRS) molecule, said method comprising the steps of:
 - a) adding a candidate bioactive agent to a sample of the IRS molecule; and
 - b) determining the binding of said candidate agent to said IRS molecule.
2. A method for screening for a bioactive agent capable of modulating the activity of an IRS molecule, said method comprising the steps of:
 - a) adding a candidate bioactive agent to a cell; and
 - b) measuring the activity of the IRS molecules in said cell.
3. A method according to claim 2 further comprising
 - c) measuring the activity of the IRS molecules in said cell prior to said addition of said candidate.
4. A method according to claim 2 or 3 further comprising
 - d) comparing the activity of the IRS molecules after addition of said candidate to the activity prior to said addition;
wherein if said activity after addition is different than the activity before said addition, said candidate is capable of modulating the activity of the IRS molecules.
5. A method of diagnosing individuals at risk for type II diabetes mellitus comprising
 - a) measuring the amount of IRS-1 in adipocytes in a first individual; and
 - b) comparing said amount to an amount of IRS-1 in adipocytes from a second unaffected individual;
wherein when the activity of IRS-1 from said first individual is less than the activity of IRS-1 in said second individual, the first individual is at risk for type II diabetes mellitus.

6. A method according to claim 1, 2, 3, 4 or 5 wherein said IRS molecule is IRS-1.

7. A method according to claim 1, 2, 3, 4 or 5 wherein said IRS molecule is IRS-2.

5 8. A method according to claim 2, 3, 4, 6 or 7 wherein said activity is determined by measuring the glucose-transport activity in response to insulin.

9. A method according to claim 2, 3, 4, 6 or 7 wherein said activity is determined by measuring the amount of the IRS molecules.

10 10. A method according to claim 2, 3, 4, 5, 6, 7 or 9 wherein said measuring is done by immunoprecipitation.

15 11. A method according to claim 2, 3, 4, 5, 6, 7, 9 or 10 wherein said measuring is done by ELISA assay.

12. A method according to claim 4 wherein said modulation comprises an increase in the amount of the IRS molecules present in said cell.

20 13. A method according to claim 4 wherein said modulation comprises an decrease in the amount of the IRS molecules present in said cell.

14. A method according to claim 2, 3, 4, 6, 7, 8, 9, 10, 11, 12 or 13 wherein said cell is an adipocyte.

25 15. A bioactive agent identified using the method of claim 1, 2, 3 or 4.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/20870

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/74 G01N33/94

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 10629 A (JOSLIN DIABETES CENTER INCORPORATED AND THE UNITED STATES OF AMERICA) 11 April 1996 see the whole document -----	1-15

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

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Information on patent family members

International Application No

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9610629 A	11-04-96	AU 3830195 A EP 0781326 A	26-04-96 02-07-97

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